THE ADJUVANT ACTION OF SERUM, EGG-ALBUMIN, AND BROTH ON TETANUS INTOXICATION.*

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IN the course of other work it became necessary to assure ourselves concerning the content in tetanus antitoxin of the sera of several goats. To our surprise normal goat serum (1.0 c.c.) rather than exerting an antitoxin action, increased the toxicity of the toxin when suitable doses of the latter were used. The investigation of this phenomenon is the subject of the present paper.

TECHNIQUE AND MATERIALS.

The toxin used throughout the experiments was prepared in January, 1903, as follows: Ten liters of beef broth containing 1 per cent of glucose and having a reaction of + 1, were placed in two bottles, and inoculated with a large quantity of tetanus bacilli which had grown for 10 days in a similar broth. Washed hydrogen was then passed through the inoculated broth for an hour, and the bottles sealed and placed in the thermostat, the usual provisions being made to permit of the escape of gas from the bottles, and to prevent the access of air. After a growth of nine days the culture was passed through Pukal filters, placed in large moisture dishes, and an excess of ammonium sulphate added;† the dishes were then placed in the thermostat over night. The brownish scum which had formed by this time was skimmed off, placed between hardened filter papers, and the excess of moisture pressed out Still more fluid and ammonium sulphate were got rid of by subjecting the precipitate to very high pressure in a pressure machine. The precipitate, now in the form of solid cakes, was dried over sulphuric acid and eventually pulverized. It is preserved over sulphuric acid in the ice-chest and in the dark.

For use a 0.2 per cent solution of the precipitate was made in 0.85 per cent sodium chloride solution, and the doses used are expressed in cubic centimeters of this solution. The original fatal

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† One-half more than the quantity the broth would dissolve at room temperature.

dose for white mice of about 15 grams weight was 0.000,007 c.c. per gram of mouse; death occurred in four to five days. For guinea-pigs of 250 to 300 grams, 0.000,001 c.c. per gram killed in four to five days. The toxin has undergone little or no deterioration since it was prepared.

When dealing with a substance of such high toxicity it is difficult to weigh out small quantities with desirable accuracy and at the same time observe economy of material. Our toxin solutions were usually made by dissolving 0.2 grams of the precipitate in 100 c.c. of the salt solution, but, in spite of great care in weighing, variations in the toxity of different solutions frequently came to light. Some of these variations may have been due to an irregular distribution of the toxin in the precipitate. A new toxin solution was prepared for each experiment.

White mice were used as the test animals throughout the work, and the dosage of toxin was based on the gram-weight of the animals—i. e., so much toxin per gram. It was found, however, that this method could be used safely only when the various mice of a given experiment were approximately of the same weight, since, for example, 0.000,007 c.c. of toxin per gram is not equally toxic for a 20 gram and a 10 gram mouse. Susceptibility is not directly proportional to weight.

The injections were made into the loose subcutaneous tissue of the back.

We soon learned that the ability of normal serum to increase the toxicity of the toxin disclosed itself only when suitable doses of the toxin were used. If a dose of toxin which killed all controls in 48 to 72 hours was used, the increased toxicity caused by the addition of serum did not become manifest. Also if the dose was so small that very little tetanus resulted in any of the animals, the effect of the serum was not always a decisive one. The clearest results were obtained when a dose of toxin was used which either caused moderate tetanus in the controls with eventual recovery, or which caused their death in from eight to twelve days.

Because of the possibility of variations in the toxicity of toxin solutions, one could not feel sure that a single dose selected for an experiment would be the optimum dose for the manifestation of the phenomenon. Hence it was decided to use a number of mice for

each experiment, and to vary the dosage in such a way that an optimum dose would be administered to two or more of the animals. It was necessary to use an abundant number of controls.

The sera of a number of normal goats were used, also of normal rabbits, and for specific purposes which will be explained later, the influence of broth and of egg-albumin was tested. Customarily 1 c.c. of the serum (or broth, or egg-albumin) was mixed with a dilution of the toxin of which 0.5 c.c. contained the desired dose. The mixture was allowed to stand for varying lengths of time and the total quantity (1.5 c.c.) then injected into the subcutaneous space in the back of the white mouse by means of a Luer or Roux syringe. After the mixture had been injected an additional 0.5 c.c. of salt solution was drawn into the syringe, and, after rinsing, was injected. In order to prevent any escape of fluid after its injection, the needle puncture was clamped for 15 to 30 minutes, the clamp being applied before the needle was withdrawn.

In all experiments daily observations were made of the degree of tetanus present, as indicated by the amount of deformity and the general appearance of the animals. The degree of tetanus is expressed in the tables by figures; thus 0 = no tetanus, 1 = perceptible rigidity; 2 = distinct but not pronounced; 3 = marked rigidity; 4 = severe; 5 = very severe to moribund; † = death. The observations of one of us served as a check on those of the other.

EXPERIMENTS.

Tables 1, 2, and 3, which follow, are typical experiments showing the influence of normal goat and rabbit sera.

TABLE 1.

THE INFLUENCE OF NORMAL GOAT SERUM ON TETANUS INTOXICATION.

Normal serum, Goat 1.—Serum three days old, unheated, preserved in the ice-chest. Serum and toxin mixed and allowed to stand 30 minutes at 37 C.° before injection.

MOUSE	WT. IN GRAMS	TOXIN, PER GRAM	C.C. OF SERUM	RESULT BY DAYS											
				1	2	3	4	5	6	7	8	9	10	11	15
1.....	14	0.000,001	1.0	0	1	2	1	1	1	1	1	1	1	1	0
2.....	14	0.000,001	0.0	0	0	1	1	1	1	1	1	0	0	0	0
3.....	16	0.000,003	1.0	0	0	2	2	2	2	1	0	1	1	1	0
4.....	10	0.000,003	0.0	0	1	2	2	1	1	1	1	0	0	0	0
5.....	14	0.000,006	1.0	0	2	3	4	4	5	†					
6.....	14	0.000,006	0.0	0	1	1	1	1	1	1	0	0	0	0	0
7.....	14	0.000,008	1.0	0	1	4	†								
8.....	14	0.000,008	0.0	0	2	2	3	3	4	3	3	3	2	1	0
9.....	13	0.000,01	1.0	0	3	5	†								
10.....	13	0.000,01	0.0	0	1	2	3	3	3	1	1	1	1	0	0

TABLE 2.

Same as Table 1, except that the serum of Goat II was used.

Mouse	Wt. in Grams	Toxin per Gram	c.c. of Serum	Result by Days												
				1	2	3	4	5	6	7	8	9	10	11	12	15
1	14	0.000,001	1.0	0	1	1	1	1	1	1	1	0	0	0	0	0
2	14	0.000,001	0.0	0	0	1	1	1	1	1	1	0	0	0	0	0
3	15	0.000,003	1.0	0	2	3	4	4	4	4	5	†				
4	15	0.000,003	0.0	0	1	1	1	2	3	3	3	2	2	2	1	0
5	14	0.000,006	1.0	0	1	3	3	4	4	4	4	4	†			
6	14	0.000,006	0.0	0	1	3	3	2	2	1	1	1	2	2	1	0
7	14	0.000,008	1.0	0	2	3	5	†								
8	14	0.000,008	0.0	0	1	3	3	4	5	†						
9	14	0.000,01	1.0	0	3	5	†									
10	14	0.000,01	0.0	0	2	2	3	3	3	2	2	2	(lost)			

TABLE 3

THE INFLUENCE OF NORMAL RABBIT SERUM ON TETANUS INTOXICATION.

Serum, unheated, two days old. Mixtures stood for one and one-half hours at room temperature before injection.

Mouse	Wt. in Grams	Toxin per Gram	c.c. of Serum	Result by Days						
				1	2	3	4	5	6	7
1	13	0.000,003	1.0	0	2	3	3	5	†	
2	11	0.000,003	0.0	0	2	3	3	4	5	†
3	16	0.000,006	1.0	0	3	4	†			
4	15	0.000,006	0.0	0	3	4	5	5	†	
5	19	0.000,008	1.0	0	4	†				
6	16	0.000,008	0.0	0	2	2	4	†		

Tables 1, 2, and 3 require little or no comment. Particularly in Tables 1 and 2, there can be no question concerning the ability of the serum to increase the intensity of the tetanus intoxication from which death occurred. Table 3 is somewhat less decisive, but here also all animals receiving serum died from one to three days in advance of the controls.

TABLE 4.

TO DETERMINE THE MINIMUM QUANTITY OF GOAT SERUM WHICH INTENSIFIES THE INTOXICATION.

Fresh serum, 18 hours old, from a normal Angora goat.

Mouse; Wt. 10 Grams	Toxin per Gram	c.c. of Serum	Result by Days					
			1	2	3	4	5	6
1	0.000,008	0.0	0	0	2	4	5	†
2	0.000,008	0.0	0	2	3	4	5	†
3	0.000,008	0.01	0	1	3	†		
4	0.000,008	0.05	0	2	3	†		
5	0.000,008	0.10	0	2	†			
6	0.000,008	0.40	0	1	3	5	†	
7	0.000,008	0.70	0	1	1	3	5	†
8	0.000,008	1.00	0	1	1	3	†	

In accordance with Table 4, 0.1 c.c. of serum is a more powerful adjuvant than 1 c.c., the latter being the dose used uniformly. This point was determined only after the major part of the experiments had been completed. Its explanation is by no means clear. The serum may contain a minute amount of antitoxin, which declares itself when larger quantities are used.

Experiments of which Table 5 is an example, were performed to determine whether the effect of the serum could be attributed to its toxic action on the mice. If such a toxic action were present, the early death of the animals might be referred to a summation of the intoxication by the serum and that by the toxin. We looked on the subsequent weight of the animals as a clue to the presence or absence of serum intoxication.

TABLE 5.

TO DETERMINE THE TOXICITY OR NON-TOXICITY OF NORMAL GOAT SERUM FOR WHITE MICE.
Serum three days old, unheated.

MOUSE	WT. IN GRAMS	C.C. OF SERUM	WEIGHT BY DAYS				
			1	2	4	5	6
1	17	1.0	18	19.5	18	18	18
2	14	1.5	15	17.0	16	15	15
3	17	2.0	18	20.0	20	18	18

There was no loss of weight, but even a gain on the second day, which may have been due to the drinking of more water. Hence we conclude that the phenomenon cannot be explained by any manifest toxicity of the serum for the mice.

Other experiments were performed with the hope that some light might be thrown on the nature of the phenomenon. It occurred to us that tetanus toxin might be an amboceptor, and that the normal serum of the goat might contain suitable complement which, when injected, would increase the amount of suitable complement in the body of the mouse, and thus activate the toxin more quickly and more completely. To throw possible light on this point the comparative influence of fresh, old, and heated sera was determined (Table 6).

The experiment shows distinctly that the effect of the serum does not depend on an activation of hypothetical tetanus amboceptors by ordinary thermolabile complement. The heated and old

TABLE 6.

THE COMPARATIVE INFLUENCE OF FRESH, HEATED, AND OLD SERA ON TETANUS INTOXICATION.

The sera were from Goat IV (female); the "heated" serum had been placed at 56° C. for 30 minutes and was freshly drawn; the "old" serum had been drawn 11 days previously and kept at about 10° C.

MOUSE	WT. IN GRAMS	TOXIN PER GRAM	1 C.C. OF SERUM	RESULT BY DAYS												
				1	2	3	4	5	6	7	8	9	10	11	14	19
1	18	0.000,006	Fresh	0	1	2	3	4	4	..	5	†				
2	18	0.000,006	Heated	0	1	2	3	3	3	..	5	†				
3	18	0.000,006	Old	0	1	2	3	4	†							
4	18	0.000,006	None	0	1	2	3	3	3	..	4	3	..	2	3	0
5	18	0.000,008	Fresh	0	0	3	3	4	4	†						
6	18	0.000,008	Heated	0	1	3	4	†								
7	18	0.000,008	Old	0	1	3	†									
8	18	0.000,008	None	0	1	2	3	3	3	..	4	3	..	2	1	0

sera were rather more efficient than the fresh serum. Serum heated even to the coagulating point does not lose its adjuvant property (Table 7).

TABLE 7.

HEAT RESISTANCE OF THE ADJUVANT SUBSTANCE.

MOUSE; WT. 12 GRAMS	TOXIN PER GRAM	1 C.C. GOAT SERUM HEATED TO —° C. FOR 30 MIN.	RESULT BY DAYS							
			1	2	3	4	5	6	7	8
1	0.000,007	67.5–68*	0	1	3	†				
2	0.000,007	67.5–68	0	3	†					
3	0.000,007	64.5–65	0	1	3	†				
4	0.000,007	64.5–65	0	1	4	†				
5	0.000,007	59.5–60	0	1	3	†				
6	0.000,007	59.5–60	0	3	†					
7	0.000,007	Unheated	0	1	4	†				
8	0.000,007	Unheated	0	1	4	†				
9	0.000,007	No serum	0	0	1	2	2	2	1	0
10	0.000,007	No serum	0	0	1	1	1	1	0	0

*The serum at 67.5°–68° was gelatinous. Temperatures higher than 68° C. were not tried.

Other experiments seem to show that the influence of the serum does not depend on any action on the toxin itself. It is not necessary to mix the serum and toxin before injection in order to get the effect of the former. The two may be injected in different parts of the body, or the serum may be injected shortly in advance of the toxin. It is well known, on the other hand, that a substance which acts directly on the toxin (tetanus antitoxin) has a stronger effect when the two are mixed before injection. In order to get the maximum effect of the serum, however, it is necessary that the two substances be injected in fairly close sequence, although they may be placed in different parts of the body (Table 8).

TABLE 8.

THE EFFECT OF THE SERUM WHEN INJECTED AT DIFFERENT PERIODS IN ADVANCE OF THE TOXIN.

1 c.c. SERUM HOURS IN ADVANCE OF TOXIN	MICE 10 GRAMS WEIGHT	TOXIN PER GRAM	RESULT BY DAYS											
			1	2	3	4	5	6	7	8	9	10	11	12
46 hours	No. 1	0.000,008	0	2	2	3	4	4	3	3	..	4	..	†
21 "	" 2	0.000,008	0	3	4	4	4	5	5	5		†		
10 "	" 3	0.000,008	0	3	3	4	5	†						
5 "	" 4	0.000,008	0	3	4	4	4	5	†					
Simultaneous	" 5	0.000,008	0	3	4	†								
No serum	" 6	0.000,008	0	2	2	3	3	4	4	4	..	5	..	†

The results of this experiment give us the impression that the action of the serum depends on some temporary influence which it exerts on the tissues of the animal, whereby the latter is made more susceptible to the toxin. After the injection of the serum its influence gradually becomes less, and is not demonstrable after the lapse of about 46 hours.

It is equally important from the standpoint of interpretation to know whether the serum intensifies intoxication when given subsequent to the injection of the toxin. The result of a single experiment indicates that the serum does not hasten the death of the animals when it is given subsequent to the binding of the toxin by the tissues. The controls which received serum and toxin simultaneously died in five days, while animals in which the injection of the serum was given from one to fifty hours later than that of the toxin either recovered or died in from six to nine days.

We had come to believe at this time that there was nothing of a specific nature in the phenomenon, and that many other proteid-containing substances might have a similar influence. We found this to be strikingly true in the cases of egg-albumin and broth (Tables 9 and 10.)

We have before us then the following facts upon which we may attempt to base conclusions:

1. The normal sera of the goat and rabbit intensify and hasten the course of tetanus in white mice, when suitable doses of our precipitated toxin are used (Tables 1, 2, and 3).

2. It is immaterial whether the serum is fresh, old, or heated to the coagulating point (Tables 6 and 7).

3. The effect is most pronounced when the serum is injected

TABLE 9.

THE INFLUENCE OF EGG-ALBUMIN ON TETANUS INTOXICATION.
One per cent of egg-albumin in 0.85 per cent NaCl solution.

MOUSE	WT. IN GMS.	TOXIN PER GRAM	C.C. OF ALBUMIN	RESULT BY DAYS								
				1	2	3	4	5	6	7	8	9
1	15	0.000,005	0.5	0	1	2	3	4	†			
2	15	0.000,005	0.0	0	0	0	0	1	1	0	0	0
3	15	0.000,006	0.5	0	1	2	5	†				
4	15	0.000,006	0.0	0	0	0	1	1	1	1	1	0
5	15	0.000,007	0.5	0	1	3	†					
6	15	0.000,007	0.0	0	0	1	1	1	1	1	0	0

TABLE 10.

THE INFLUENCE OF BROTH ON TETANUS INTOXICATION.

MOUSE	WT. IN GMS.	TOXIN PER GRAM	C.C. OF BROTH	RESULT BY DAYS										
				1	2	3	4	5	6	7	8	9	10	11, 12, 13
1	15	0.000,006	1	0	0	3	3.	4	†					
2	15	0.000,006	0	0	0	1	2	3	3	4	4	4	3	Recovery
3	18	0.000,007	1	0	1	3	†							
4	18	0.000,007	0	0	1	2	3	4	5	†				
5	15	0.000,008	1	0	1	†								
6	15	0.000,008	0	0	1	2	4	†						

simultaneously with, or shortly preceding, the injection of the toxin. If the serum is given 46 hours in advance of the toxin it is in some way disposed of so that it no longer intensifies the intoxication. It is not necessary that the serum be mixed with the toxin, nor injected into the same part of the body (Table 8).

4. A small quantity of serum (0.1 c.c.) seems to have a more pronounced influence than a larger quantity (1 c.c.) (Table 6).

5. Normal goat serum in a quantity of 2 c.c. produces no perceptible deleterious effect on the mouse (Table 5).

6. One-half c.c. of a 1 per cent solution of egg-albumin in physiologic salt solution; and also 1 c.c. of broth have an influence like that of serum.

The possibility which we at one time took under consideration that tetanus toxin might be an amboceptor, and that the serum increases toxicity because it provides anadditional quantity of complement cannot be entertained: first, because old and heated serum, in which there is no complement (thermolabile), produce the phenomenon; and, second, because egg-albumin or broth may be sub-

stituted for the serum with the same result. We cannot accuse the broth, in particular, of containing complement.

Inasmuch as we have not been able to refer the phenomenon to any influence which the adjuvant substances may exert on the toxin, we have been obliged to assume that it is due to some effect on the tissues of the mice. Although the serum exerted no perceptible toxic action on the mice, which if it had occurred might have lessened resistance in some manner, we were bound to consider as a possibility that the serum, albumin, and broth might exert some particular influence on the nervous tissue whereby it either absorbed more toxin or was made less resistant to the toxin which it bound. In spite of this possibility, however, we have been unable to conceive of any manner in which these substances could produce such an effect on the nervous tissue. If the serum, etc., were to some extent bound as indifferent food substances by the nervous cells, we believe the latter might be preoccupied, so to say, in digesting them; but since such a process would engage the cells in a general way we believe their affinity for some second substance, such as tetanus toxin, would be decreased rather than increased during this period. Concerning the alternative possibility that the serum, etc., may injure the nervous cells so that they are less resistant to the toxin, we have only the argument that no injury detectable by gross means was produced.

We find what appears as a more reasonable explanation of the phenomenon in an influence which the serum, etc., may exert on the remaining tissues of the body other than the nervous tissue. We learn from certain investigations by Metchnikoff,[1] and by Roux and Borrel,[2] that other tissues than the nervous are able to bind tetanus toxin in some instances. The rabbit and also the chicken are much more susceptible to tetanus toxin when it is injected into the nervous tissue than into the subcutaneous tissue; in the latter instance the toxin comes in contact with various tissues some of which bind a certain amount. Metchnikoff found that the liver in some of the invertebrates absorbs a great deal of tetanus toxin. We are not aware of any experiments bearing on the antitoxic powers of the organs of the white mouse, nor have we performed

[1] *L'Immunité*, Paris, 1901, p. 343.

[2] *Ann. de l'Inst. Pasteur*, 1898, 12, p. 229.

such experiments. Wasserman and Takaki found that small quantities of the liver, kidney, spleen, and bone-marrow of the guinea-pig exerted no antitoxic action, a condition which corresponds well with the exquisite susceptibility of this animal to tetanus. In comparing the susceptibility of the rabbit with that of the guinea-pig, we learn, first, that, gram for gram, the rabbit is about a thousand times more resistant than the guinea-pig (Knorr, cited by Dieudonné[1]); and second, from Roux and Borrel, that a large part of this resistance resides in other than nervous tissues. The *tetanus sine tetano* of Dönitz suggested to him that non-nervous organs in the rabbit bind tetanus toxin.[2] Such animals after receiving a dose of toxin which causes little or no tetanus gradually become emaciated and die.

We cannot of course hold, without definite experimental proof, that the same conditions exist in the mouse. We know, however, that the mouse, gram for gram, is from six to ten times more resistant to tetanus toxin than the guinea-pig (Knorr[3]); in our own experiments it is seven times more resistant, and it is quite possible that some of this resistance depends on the ability of tissues other than the nervous to bind a certain amount of the toxin.

If this condition exists, and if in some way union of the toxin with other tissues could be prevented, for example, with the liver, connective tissues, or lymphoid organs, just so much more toxin would be available for the more susceptible nervous tissue.

We conceive that such a result may be caused by the serum, etc., in one of two ways: First, certain receptors or substances in the serum, egg-albumin, and broth, may possess cytophilous haptophores, identical with that of tetanus toxin, and by uniting with the tetanophile receptors of indifferent organs (liver, etc.) may thereby render impossible the binding of the toxin; this extra toxin would then be available for the nervous tissue, which presumably has a higher affinity than other tissues for the toxin. Such a process would consist of a preoccupation of tetanophile receptors by heterologous substances.

Second, the cells of indifferent organs (liver, etc.) may bind the serum, etc., as they would bind food substances, after which their

[1] *Immunität, Schutzimpfung, etc.*, Leipzig, 1903, p. 13. Cited by Dieudonné, *loc. cit.*

[2] *Deutsche med. Wchnschr.*, 1897, 23, p. 428.

activities (ferments) may be directed toward the digestion or oxidation of the new substances, and being thus engaged, the affinities of their receptors for other substances (toxin) may be decreased. This condition would also render more toxin available for the nervous tissue. Such a process would consist of a non-specific engagement of the activities of the cells, without a direct occupation of tetanophile receptors.

As an example of such a process we may mention the well-known experiments of Besredka,[1] in which granules of carmin injected into the peritoneal cavity decreased the ability of the leucocytes to take up granules of arsenic, with the result that the animals died the more readily of arsenic intoxication.

Von Dungern also observed a clear example of the ability of one substance to interfere with the absorption of a second.[2] By injecting the plasm of *Octopus vulgaris* or egg-albumin into the circulation of a rabbit the ability of the animal to absorb a subsequent injection of the plasm of *Maja squinado* (spider crab) was largely or entirely inhibited. Von Dungern's explanation of this phenomenon is somewhat incomplete, but we understand it to be the following: In reference to the particular substances injected, the cells of the rabbit have two types of receptors. One type is that which is able to take up various materials as food substances and is not a specific receptor. It takes up egg-albumin, the plasm of the spider crab and of the octopus, and doubtless many other albumins. It is not concerned in the formation of antibodies. The other type is specific, can take up only one particular substance, and when it proliferates, is cast into the circulation as an antibody.

Now when the plasm of the octopus or egg-albumin was injected into the rabbit, the first type of receptor, the non-specific type, was occupied largely or completely, and hence was not able to fix the plasm of the crab which was injected two and one-half hours later. Only the second type, the specific receptor, remained to fix the plasm of the crab. As a consequence the last-named substance disappeared from the circulation much more slowly than when it was injected into an untreated rabbit.

[1] *Ann. de l'Inst. Pasteur*, 1899, 12, pp. 49 and 209. [2] *Die Antikörper*, Jena, 1903, p. 97.

We interpret the phenomenon which we have described in a somewhat similar way, although it is necessary for us to introduce the consideration that, the tissues of the mouse having united with the serum, egg-albumin, or broth, they thereby lose in their affinity for tetanus toxin. After the foreign substances have been disposed of by the cells, the latter again reach a state of equilibrium, and their tetanophile receptors regain their former affinity for the toxin (Table 8).

It is not possible to assume, on the basis of our experiments, that an antitoxic or bactericidal serum can in any way intensify the corresponding disease, provided the serum is sufficiently rich in antibodies. In diphtheria, for example, the quantity of antitoxin introduced is able to neutralize all the toxin which happens to be in the body, hence there is no possibility of intensifying the diphtheritic intoxication.